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14. ABSTRACT
We have made further progress toward preparing lead compounds for new anticancer drugs from a novel class of starting materials containing the cyclopentenone scaffold. These compounds are inspired by natural products with proven anti-cancer and anti-viral activities. We have prepared parallel library #2 through a variation of the general chemical methodology that was used to prepare library #1 (see last year's report). This new cyclopentenones comprise a library of complex, polyfunctional organic molecules of unprecedented structure. The most important class of enzymes for biocatalytic amplification of these compounds is the cytochrome P450s. We have developed new reaction systems (e.g., surfactant-stabilized microemulsions and hydrogel-entrapped enzymes in organic solvents) that will expand the synthetic utility of cytochrome P450s and render them much more effective catalysts for structural elaboration of the chemically synthesized compounds. We have also begun to screen our libraries against several cell lines including solid tumor, leukemia, and normal cells. Combining combinatorial synthesis with biocatalytic amplification of chemical libraries is a new approach to drug discovery, which we are applying to a promising but largely unexplored class of compounds. Even if we do not identify a new clinical candidate, it is becoming increasingly likely that we will identify lead compounds.

15. SUBJECT TERMS
Parallel Synthesis: Biocatalytic Amplification; Drug Discovery; Chemotherapeutics; Lead Optimization

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INTRODUCTION

The traditional approach to cancer drug discovery is to survey the natural world, either collect plant material or grow microorganisms, and determine whether any of the large number of compounds that is present has the ability to arrest the growth and proliferation of cancer cells. The newer approach is to use the tools of chemical synthesis to create large collections ("libraries") of organic molecules, with the hope that one or more of them will hold promise for the treatment of cancer. We propose to *combine* the traditional natural products-based and library-based approaches to cancer drug discovery into a single hybrid approach that incorporates the power of chemical synthesis and biocatalysis. We will use a combination of highly efficient chemistry and biocatalysis to prepare a library of small organic molecules whose structures are inspired by natural products known to be active against cancer. Thus, the primary objective of this project is to combine the two traditional paradigms for drug discovery into one, thereby enabling a more effective and efficient route to the advent of new chemotherapeutics against breast cancer.

BODY

Research accomplishments are presented below in connection with each task outlined in the original Statement Of Work. Previous progress on Tasks 1-3 is described in last year's annual report.

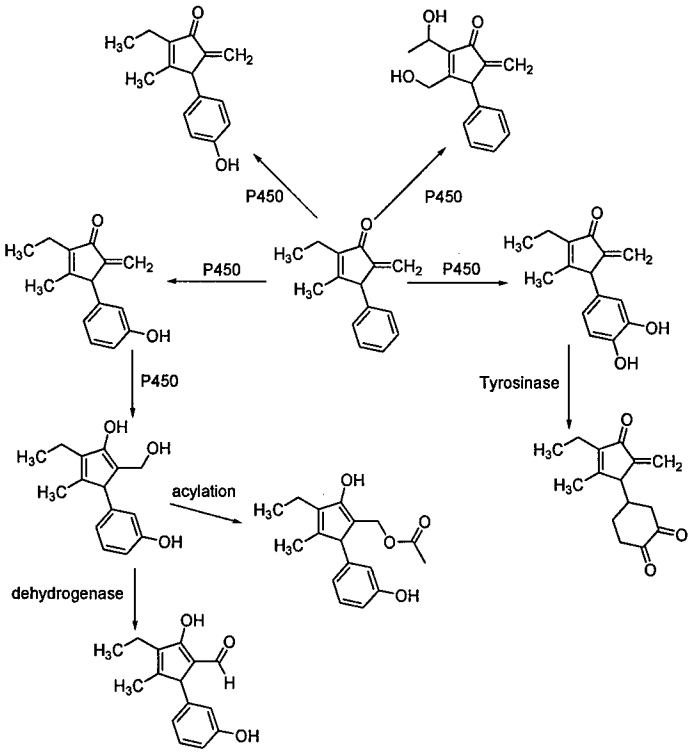
Task #2, #4, #6, and #8

With the help of Dr. Fred Valeriote of the Henry Ford Health System, we have begun to screen our libraries against several cell lines including solid tumor (colon 38, colon H116, and lung H125), leukemia (L1210 or CEM), and normal (CFU-GM) cells. While the results are still quite preliminary, one compound showed some selectivity for the human solid tumors versus human leukemia. Our screening efforts against these and other cell types (e.g., MCF7 breast cancer cells) will continue and expand during the final phase of the project.

Task #3 and #7

Developing two-phase and nonaqueous reaction systems for P450-catalyzed transformations

The model cyclopentenones are very hydrophobic molecules, and the biocatalytic formation of potential products is limited by the solubility of these molecules in aqueous solution. In particular, P450-catalyzed oxidations represent an extremely powerful class of reactions for amplifying the cyclopentenone libraries and thereby generating lead compounds with promising anti-cancer activities. Not only do P450s introduce oxygen atoms that can enhance bioactivity by increasing the hydrophilicity and/or physicochemical reactivity of the molecule, P450-catalyzed hydroxylations generate functional handles for further derivatization by chemical or biocatalytic transformations (potential P450-catalyzed transformations of a representative cyclopentenone are shown below).



Our initial efforts to expand the cyclopentenone libraries by P450-catalyzed reactions—although encouraging--were hindered by limited substrate solubility. Therefore, we set out to develop reaction systems that would enable P450s to function in either non-aqueous solvents or in two-phase aqueous-organic mixtures. To this end, a model P450, P450cam, has been cloned, expressed, and purified.

The activity of P450cam has been investigated in two two-phase systems: a surfactant (AOT) stabilized microemulsion, and polyvinyl alcohol/polyethylene glycol hydrogels suspended in hexane. The activity of P450cam toward the oxidation of camphor was assayed for both systems. The formation of the product, hydroxycamphor, was monitored using gas chromatography for reactions containing varying amounts of camphor. Results from experiments in surfactant-stabilized microemulsions were very encouraging and are shown in Figure 1. With the addition of yeast alcohol dehydrogenase (YADH) and ethanol to regenerate the cofactor, NADH, near-

complete conversion of camphor into hydroxycamphor was achieved. The high product yields obtained with P450cam in two-phase emulsions are at least four-fold greater than any previously reported results. The next step will be to determine whether similar yields can be obtained using P450cam and other P450s, e.g., P450 BM-3, and high concentrations of cyclopentenone substrates.

An alternative reaction system, which may afford more stable and reusable P450 catalysts, consists of P450s entrapped in hydrogels. P450cam was thus entrapped in PEG/PVA hydrogels and its activity was demonstrated both in aqueous and non-aqueous solvents. To our knowledge, this is the first example of entrapping a functional P450 in a hydrogel for biocatalysis in either an aqueous or organic solvent.

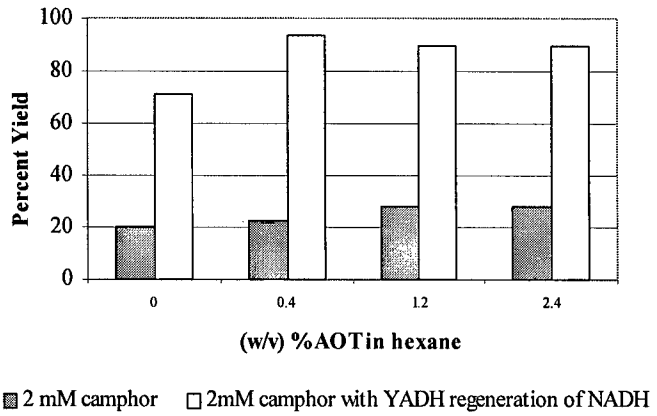
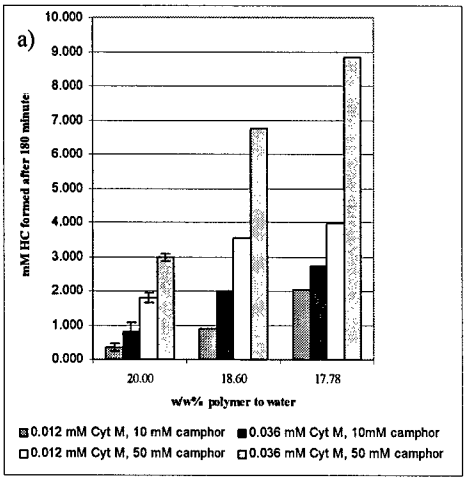


Figure 1. P450cam-catalyzed conversion of camphor to hydroxycamphor in AOT-stabilized water/hexane microemulsions.

The maximum amount of hydroxycamphor that can be produced in an aqueous system is limited by the solubility of camphor in water, which is 1.2 mM. However, because of increased camphor solubility in hexane, the formation of hydroxycamphor catalyzed in hexane by the P450cam/hydrogel exceeded that which is possible in an aqueous system. Figure 2 shows endpoint determinations of hydroxycamphor production after 180 minutes by P450cam/hydrogel beads containing either 0.012 mM or 0.036 mM P450cam in the presence of either 10 or 50 mM camphor. Note that the activity of the P450cam/hydrogel catalyst increased with increasing water content.



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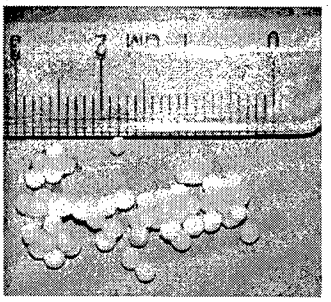


Figure 2. a) Activity of P450cam in hydrogels with increasing water content. The production of hydroxycamphor by each of the gels was measured after 180 minutes. b) Hydrogel beads (1.5 mm in diameter) containing P450cam.

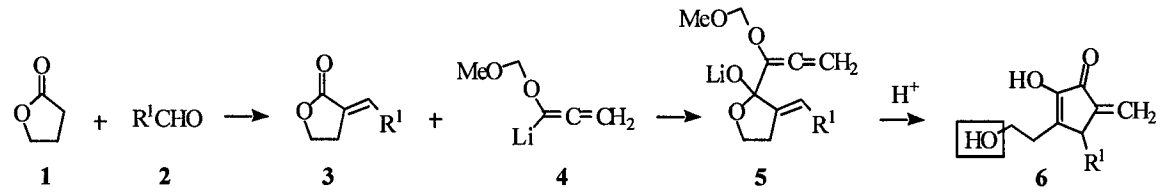
In conclusion, high activity of P450cam has been demonstrated in two predominantly nonaqueous reaction systems. This is an unprecedented achievement that bodes well for biocatalytic amplification of the hydrophobic cyclopentenone libraries, and is a major step toward using P450s and other monooxygenases for more general synthetic applications.

Task #5

We have successfully prepared parallel library #2 through a variation of the general chemical methodology that we have used in the preparation of library #1. We were motivated to explore a method that would allow us to prepare cyclopentenones bearing hydrophilic functionality, i.e. one or more additional hydroxyl groups. We felt that it would be very useful to explore compounds in which the hydrophilicity has been modulated, since nearly all the materials in library #1 had been to various degrees lipophilic. Transport (and other) properties for the lipophilic vs. the hydrophilic groups of compounds could be quite different, therefore if we ignored one of the two groups, we risked missing activity.

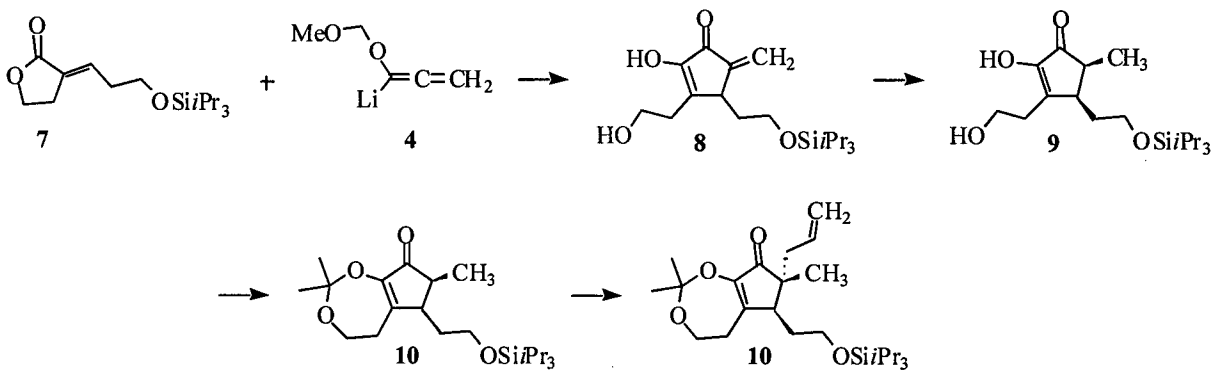
There are several ways in which we could have implemented this goal. One approach would have been to introduce the hydroxyl group(s) *subsequent* to the cyclization. A simpler solution to the problem is to use a lactone, specifically an alkylidene lactone, in place of the morpholino enamides that we normally use. The lactone incorporates the additional hydroxyl group, but in an internally protected form. At the outset it was not at all clear whether this chemistry would work.

Scheme 1



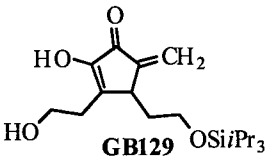
Scheme 1 summarizes our approach. Commercially available cheap butyrolactone **1** was condensed with aldehydes **2** to give a-alkylidene butyrolactones **3**. These were condensed with allenyllithium reagent **4** to give the presumed tetrahedral intermediate **5**. Premature collapse of **5** to a ketone would be problematic, since such an event would probably lead to a variety of undesired reactions with excess **4**. Fortunately this did not happen, and conventional workup with aqueous acid led to cyclopentenones of general structure **6** in good yield. To date we have only explored the use of butyrolactones. Valerolactoones and caprolactones should also work. The significant feature of **6** is its amphiphilic nature. The "left hand" portion of the molecule incorporates two hydroxyl groups and is consequently hydrophilic, whereas the "right hand" portion bears only hydrocarbon functionality and is lipophilic. It is significant that no protecting groups are needed, since that would have added to the labor involved in the preparation.

Scheme 2



The next task was to apply this chemistry toward the synthesis of a series of structurally related cyclopentanoids that would become the kernel of library #2. The chemistry is summarized in Scheme 2. Alkylidene butyrolactone **7** was condensed with allenyllithium **4** to produce **8** upon treatment with aqueous acid during workup. Selective saturation of the exocyclic C-C double bond in **8** leads to **9**. The diol function in **9** is easily converted to the acetonide, leading to **10**. Exposure of **10** to lithium diisopropylamide followed by allyl bromide gives **11**. Compounds **8-11** form a homologous series that makes it possible to dissect the parts of the parent structure (e.g. **8**) that affect the cytotoxicity. For example, a comparison of the activities of **8** and **9** will reveal the effect, if any, of the exocyclic methylene group. A comparison of **9** and the derived acetonide **10** will show if hydrophilicity in one portion of the structure enhances activity. In derivative **11**, steric encumbrance to the carbonyl group has been introduced. If activity is dependent upon interactions between the carbonyl group and its target, it should be attenuated in **11** compared to **10**.

Table 1



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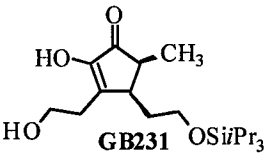

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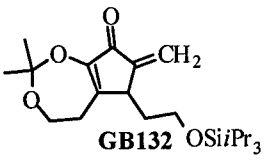

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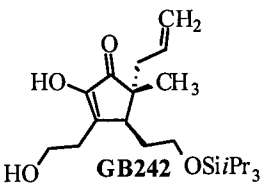

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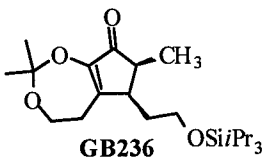

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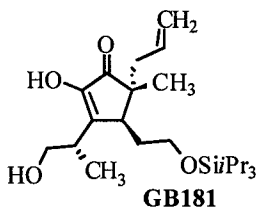

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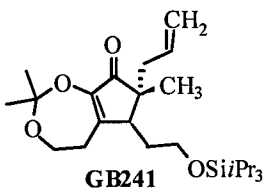

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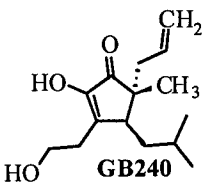

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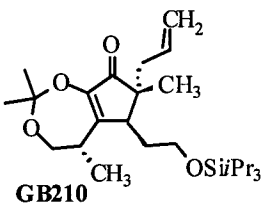

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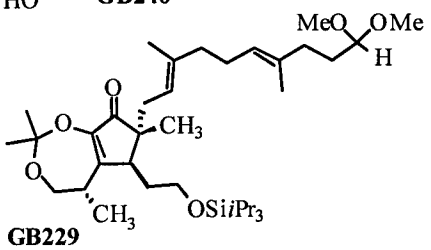

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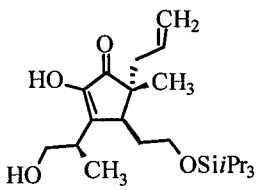

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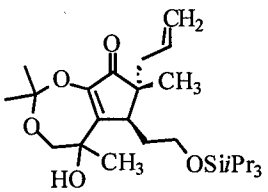

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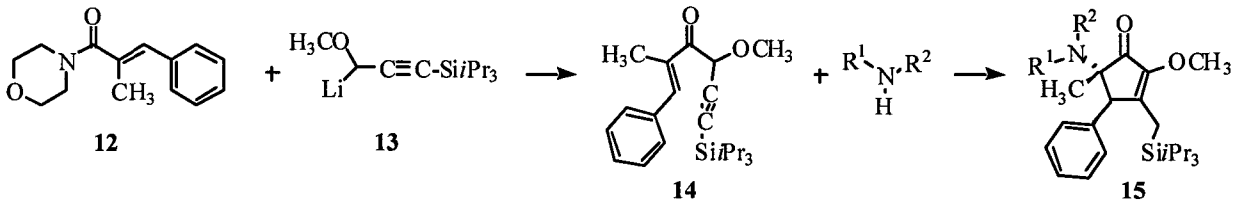
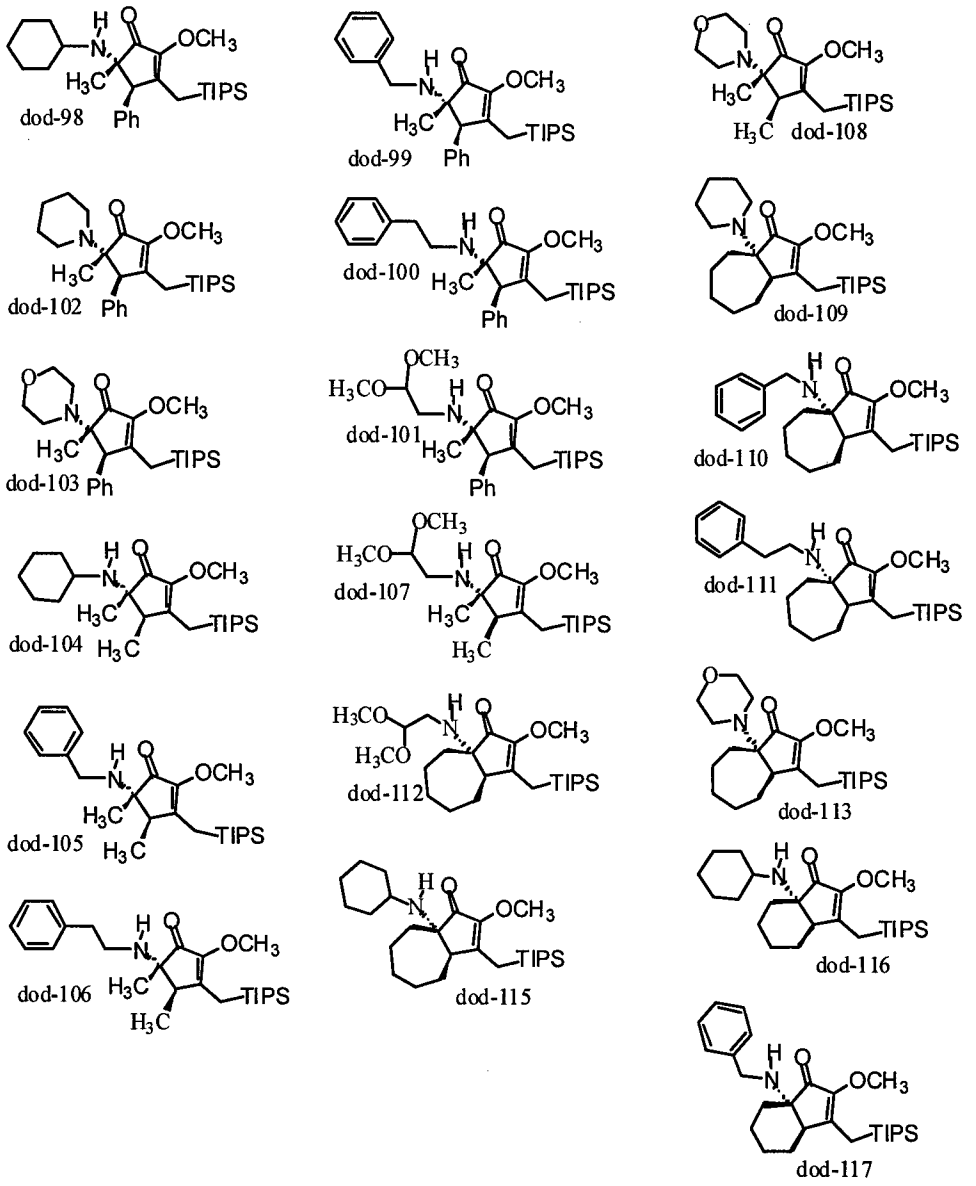
It will be recognized that the preparation of compounds **9-11** requires additional chemical steps following the cyclopentannelation reaction. This is a price worth paying, we believe, to access structures with greater molecular complexity. More complex structures have the potential to engage their biological targets in a greater variety of ways, increasing the probability of observing useful activity.

Scheme 3



We felt that it would also be highly desirable to find a way to introduce a nitrogen atom into the structures. It is a well-established fact that the presence of one or more nitrogen atoms is a feature of many useful pharmaceutical products. Incorporating a nitrogen atom into our structures would allow us to explore new biological space. Several ways to introduce nitrogen *after* cyclization were dropped from consideration early one for reasons of practicality. Work in our labs unrelated to this grant revealed an unanticipated solution to the problem that is summarized in Scheme 3. Addition of propargyllithium species **13** to morpholino enamide **12** leads to enone **14**. This compound is simply mixed with a small excess of an amine in the presence of dry silica gel (no solvent). A series of rearrangements take place at room temperature leading to α-aminoketones **15** in good yield. Table 2 lists the structures that have been prepared to date and which are currently being evaluated.

Table 2



KEY RESEARCH ACCOMPLISHMENTS OF THIS REPORTING PERIOD

Hydrophilic, molecularly complex cyclopentenones were prepared through a variation of the cyclopentannelation process.

This protocol has been used to prepare a library of complex, polyfunctional organic molecules of unprecedented structure.

A library of α-aminocyclopentenoens has been prepared and is being evaluated.

P450cam has been expressed, cloned, and purified for use in developing nonaqeuous reaction conditions for P450 monooxygenases.

The activity of P450cam has been demonstrated in a two-phase reaction system, and the product yields are over 4-fold higher than any previously reported conditions for P450cam.

The activity for P450cam entrapped in PVA/PEG hydrogels has been established. Using high substrate concentrations in the organic phase enables product concentrations that greatly exceed the solubility limits in aqueous solution.

REPORTABLE OUTCOMES

"A Tandem Alkylation-Cyclization Process via an *O,C*-Dianion." Banaag, A. R.; Berger, G. O.; Dhoro, F.; delos Santos D. B.; Dixon, D. P.; Mitchell, J. P.; Tokeshi, B. K.; Tius, M. A. *Tetrahedron* **2005**, *61*, 3419-3428.

CONCLUSIONS

We have made further progress toward preparing promising lead compounds for new anticancer drugs from a novel class of starting materials inspired by natural products with proven anti-cancer and anti-viral activities. More powerful and versatile chemical methodology and enzymatic reaction systems have been developed in the second stage of our research with the aim of producing a high degree of structural diversity from the cyclopentenone scaffold. In this connection, a library of complex, polyfunctional organic molecules of unprecedented structure has been generated. We have also expanded the groundwork for carrying out enzymatic reactions on these molecules, particularly P450-catalyzed oxidations, a pivotal first-step toward generating novel analogs in subsequent enzymatic and chemical reactions. The bar for discovery of a new anticancer drug is set very high. Nevertheless, we are developing and using a unique approach. Even if we do not identify a clinical candidate during the time of this grant, it now appears likely that we will identify several lead compounds. Perhaps one of them will exert its activity through a new mechanism of action.